Z. Klin. Chem. Klin. Biochem.
13. Jg. 1975, S. 273–276

Serum Dopamine β-Hydroxylase: Assay and Enzyme Properties

By *E. S. Markianos* and *Irene E. Nyström*

Neurochemische Abteilung der Psychiatrischen Klinik der Universität München

(Eingegangen am 8. Juli/17. Oktober 1974)

Summary: A method for the estimation of dopamine β-hydroxylase activity in human serum is described, based on a thin layer chromatographic separation of the substrate ([^{14}C]tyramine) from the reaction product ([^{14}C]octopamine). The basic properties of the human serum enzyme, investigated by this method are described.

Dopamin-β-hydroxylase im Serum: Bestimmung und Eigenschaften

Zusammenfassung: Eine Methode zur Bestimmung der Dopamin-β-hydroxylase in Serum wird beschrieben. Das Substrat, [^{14}C]Tyramin, wird dünnschichtchromatographisch vom Produkt, [^{14}C]Octopamin, getrennt. Die Auswertung erfolgt durch einen Dünnschichtchromatographie-Scanner. Die mit dieser Methode untersuchten Eigenschaften des Enzyms werden beschrieben.

Introduction

Dopamine β-hydroxylase (EC 1.14.2.1), the enzyme that catalyzes the hydroxylation of dopamine to noradrenaline, was found to be localized in the catecholamine containing vesicles of the sympathetic nerve terminals and the adrenal medulla (1). During nerve stimulation, the enzyme is released together with catecholamines (2,3) and appears in the blood as circulating dopamine β-hydroxylase activity.

The enzyme hydroxylates a number of phenylethyl- and phenylpropylamines (4). It is inhibited by a variety of chelating agents (5). The enzyme contains copper in the molecule which undergoes cyclic reduction and oxidation during the hydroxylation of the substrate (5,6). Recently, dopamine β-hydroxylase was found to be a tetrameric glycoprotein with about 4 % carbohydrates and 4 copper atoms per molecule (7).

In serum, low concentrations of copper cause a marked increase in activity (8) by removing an inhibitor. The same effect is shown by reagents that react with sulfhydryl groups. The inhibitors can build chelates with copper in situ rather than remove the metal from the enzyme (9).

Until a sensitive radioactive assay was introduced (10), methods of assay were based on the separation of the reaction product from the substrate by column chromatography. In the former, the main incubation is followed by a second one with purified phenylethanolamine-N-methyl-transferase and [^{14}C]S-adenosylmethionine. The N-methyl-phenylethanolamine formed is then extracted and counted in a scintillation counter.

A proportional release of noradrenaline and dopamine β-hydroxylase into the blood was found (11). The release occurs by exocytosis, during which a fusion of the vesicular and neuronal membrane takes place.

For the estimation of dopamine β-hydroxylase activity in nonpurified biological materials, where other enzymes acting on catecholamines are present, it is necessary to make a qualitative check of the nature of the reaction products. This was performed by using thin layer chromatographic or gas chromatographic methods for the estimation of the changes in substrate and product concentrations during the incubation.

Using [^{14}C]tyramine as substrate for the enzyme, we applied an aliquot of the incubation mixture directly to a thin layer chromatographic plate. After development, the plate was scanned and two well separated peaks of tyramine and octopamine were obtained. Integration of the peaks gives the relative amounts of substrate and product present in the incubation mixture at the time of the application on the plate.

This direct analysis of the incubation mixture has certain advantages for the exact estimation of the enzyme activity: Any eventual byproducts can be identified, the amount of substrate still present can be controlled, the analysis can be carried out without interrupting the reaction and the estimation is independent of the volume applied to the plate. Many sources of error are thus eliminated.

Materials

[2-^{14}C]Tyramine acetate (50 Ci/mol) was obtained from CEA (Commissariat a l'énergie atomique), France. Catalase was obtain-

ed from Schuchardt, Munich, Germany; octopamine from Sigma Chemical Company, U.S.A. Pre-coated Silica gel plates and the other chemicals from E. Merck, Darmstadt, Germany. Pentafluoropropionic acid anhydride for the gas chromatography was from Pierce Chemical Company, U.S.A.

Methods and Results

Thin layer chromatography

Pre-coated silica gel plates 200 × 200 mm were used. The best separation of tyramine from octopamine was achieved with a solvent consisting of chloroform, methanol, acetic acid, water (Volumes 60 ml + 25 ml + 15 ml + 5 ml).

Table 1 shows the R_F values for a number of catecholamines and their metabolites as well as tryptophan and its metabolites.

Table 2 refers to a number of enzymes which could be estimated using basically the same procedure described.

Until now, we have used this procedure successfully for the estimation of dopamine β-hydroxylase activity in serum, brain and adrenal medulla, and of tyrosine hydroxylase and tryptophan hydroxylase in brain.

Tab. 1. Thin layer chromatography of catecholamines, tryptophan and their metabolites.

Substance	R_F	Color in UV
Tyrosine	0.30	blue
Phenylalanine	0.52	blue
L-DOPA	0.16	dark violet
Dopamine	0.38	dark violet
Noradrenaline	0.20	violet
Adrenaline	0.26	violet
Tyramine	0.53	light violet
Octopamine	0.42	light violet
Metanephrine	0.51	blue
Normetanephrine	0.46	blue
3-Methoxytyramine	0.58	light violet
3,4-Dihydroxyphenylacetic acid	0.68	gray-blue
3-Methoxy-4-hydroxyphenylglycol	0.77	blue
3,4-Dihydroxymandelic acid	0.89	blue
3-Methoxy-4-hydroxymandelic acid	0.45	blue
4-Hydroxyphenylacetic acid	0.89	blue
Homovanillic acid	0.94	blue
Homovanillic alcohol	0.96	dark violet
Tryptophan	0.45	light violet
5-Hydroxytryptophan	0.23	dark violet
5-Hydroxytryptamine	0.46	brown
N-acetyl-5-hydroxytryptamine	0.78	violet
5-Hydroxy-indolacetic acid	0.98	dark violet

Assay of serum dopamine β-hydroxylase

The incubation mixture contained in a volume of 0.15 ml: citrate-phosphate buffer pH 4.80, 0.124 mol/l; ascorbate 2.0 mmol/l; fumarate 60 mmol/l; pargyline 10 µmol/l; catalase 250 units; copper sulfate 25 µmol/l; [^{14}C]tyramine 50 µmol/l. The ascorbate and fumarate solutions were adjusted to pH 5.20.

To this mixture, 50 µl serum were added and incubated at 37°C for 40 minutes. The amount of tyramine hydroxylated is, under these conditions, proportional to the incubation time for up to 80 minutes for the enzyme activities usually found in serum. The substrate consumption is usually about 20 %. 20 µl of the reaction mixture are applied to a thin layer plate, developed and the plate scanned. The scanner used was the Berthold Dünnschichtscanner, Wildbad, Germany, in combination with a scaler-timer for counting the radioactivity. The dopamine β-hydroxylase activity was expressed in U/l (micromoles tyramine hydroxylated per l serum and minute). A mean value of 1.015 ± 0.570 U/l was found from the sera of 91 hospitalized, non-psychiatric patients.

Figure 1 shows the increase of the octopamine peak with the incubation time.

Identification of the reaction product by gas chromatography

The second radioactivity peak that appears after incubation of [^{14}C]tyramine with serum, has the same R_F value as octopamine on thin layer plates.

In addition, after incubation, the incubation mixture was freeze-dried, the methanol soluble part taken and reacted with pentafluoropropionic anhydride. After evaporation of the

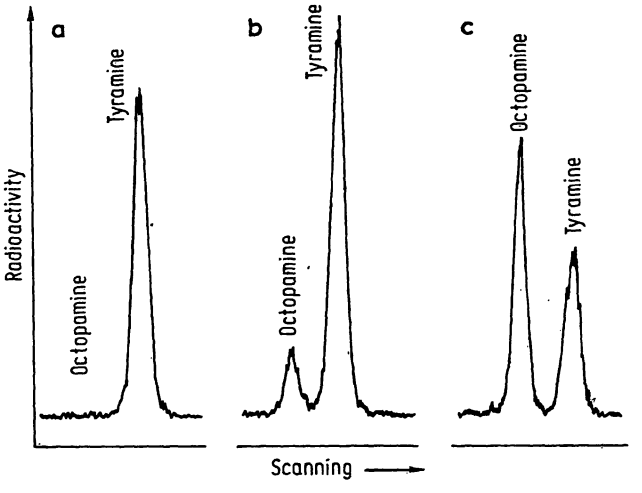

Fig. 1. Radiochromatograms of an incubation mixture for serum dopamine β-hydroxylase at time zero (a), 20 (b) and 120 (c) minutes.

Tab. 2. Enzyme activities which can be estimated using the thin layer chromatographic method described.

Enzyme	EC	Substrate	R_F	Product	R_F
Phenylalanine hydroxylase	1.14.16.1	Phenylalanine	0.52	Tyrosine	0.30
Tyrosine hydroxylase	1.14.16.2	Tyrosine	0.30	DOPA	0.16
Dopamine β-hydroxylase	1.14.17.1	Dopamine	0.38	Noradrenaline	0.20
		Tyramine	0.53	Octopamine	0.40
Tryptophan hydroxylase	1.14.16.4	Tryptophan	0.45	5-Hydroxytryptophan	0.23
Aromatic-*L*-amino-acid decarboxylase	4.1.1.28	5-Hydroxytryptophan	0.23	5-Hydroxytryptamine	0.46
		DOPA	0.16	Dopamine	0.38

reagent, the product was dissolved in ethyl acetate (dry) and injected into a gas chromatographic system (Hewlett-Packard 7620A), with a coiled glass column 6 feet × 4 mm, packed with chromosorb WAW DMCS 80–100 mesh, coated with 6 % OV-17. Carrier gas flow rate 40 ml/h (helium), oven temperature (isotherm) 140°C. Electron capture detector (nickel), temperature 175°C. Argon-methane flow rate 60 ml/h.

Under these conditions, the retention time was 1176 s for tyramine and 1320 s for octopamine. The pentafluoropropionic anhydride derivatives gave a good response and were stable for days.

This gas chromatographic estimation of tyramine and octopamine, could be used for the estimation of dopamine β-hydroxylase activity in cases where very small amounts of substrate are possible, without labelled substrate being necessary (e.g. experiments in vivo).

pH optimum of the serum enzyme

Figure 2 shows the relation of dopamine β-hydroxylase activity to the incubation pH. The activity has a maximum between pH 5.2 and 5.4. Outside this pH range, great variations in activity are found. This makes it necessary to check the pH for every incubation. In the method described, after the application on the plate, the pH is measured in the remaining incubation mixture using a microelectrode.

pH stability of the serum dopamine β-hydroxylase

0.5 ml serum were kept at pH between 3 and 12 for 30 minutes at 37°C, neutralized and incubated at pH 5.2–5.4 as usual.

The enzyme shows optimum stability at physiological pH and is totally inactivated under pH 4 and over pH 11. The enzyme in blood, is under the conditions for its optimal stability, but is not active because of the high pH. The serum enzyme remains idle as long as it is in the blood.

Influence of copper concentration

Figure 3 shows the dependence of the enzyme activity on the copper concentration present in the incubation mixture. Without addition of copper, a little or no activity is found. This activity increases if the serum is kept for several days at 4°C, although the activity at 25 μmol/l copper remains the same, so that an inactivation of some inhibitor can be assumed.

Temperature stability of the serum enzyme

Serum was kept for 60 minutes at temperatures between 25°C and 68°C and then incubated at 37°C (Fig. 4).

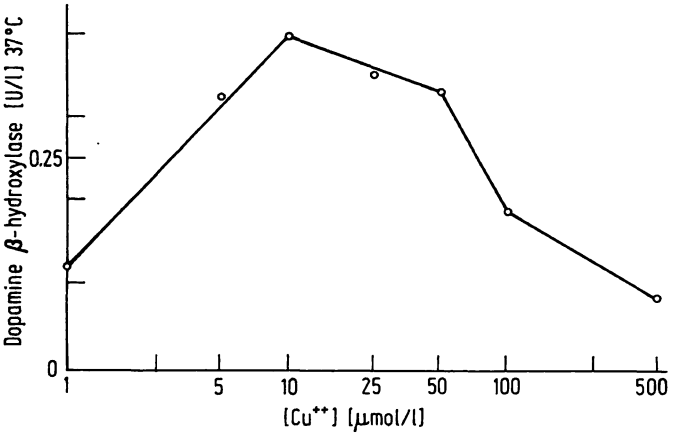

Fig. 3. Variation of serum dopamine β-hydroxylase activity with copper ions added to the incubation mixture.

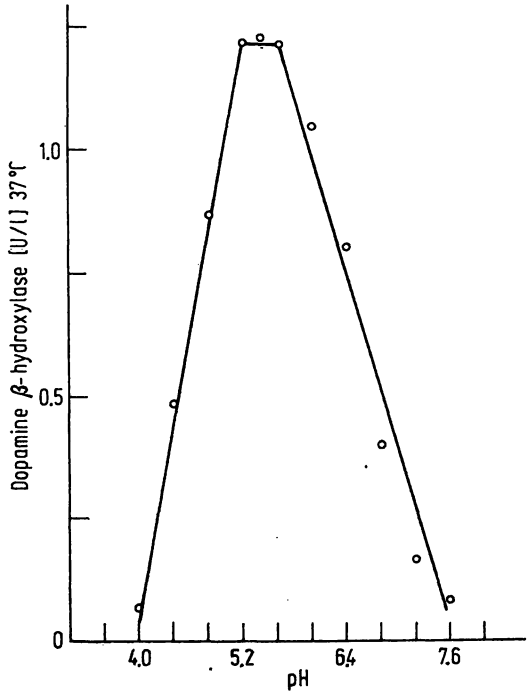

Fig. 2. pH optimum of serum dopamine β-hydroxylase activity.

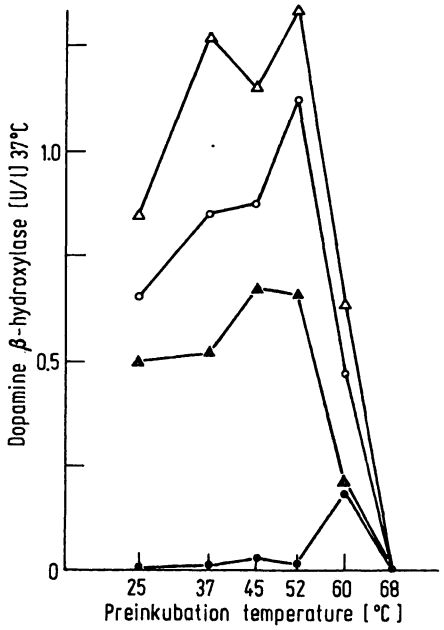

Fig. 4. Temperature stability of serum dopamine β-hydroxylase. 60 min preincubation at 25–68°C. Activity determined at 37°C in the absence (●——●) and in the presence of 5 (○——○), 25 (△——△) and 100 (▲——▲) μmol/l Cu^{++}.

The enzyme in serum is totally inactivated only at temperatures over 60°C. When incubated without addition of copper, activity appears in the serum kept at 60°C, which indicates that at least some inhibitor is inactivated before the whole enzyme is inactivated. The increase in activity at 52°C and 5 μmol/l copper, is a result of the same effect.

When the incubation temperature is varied, an increase in activity is found with temperatures up to 52°C, with a rapid fall over that temperature (Fig. 5).

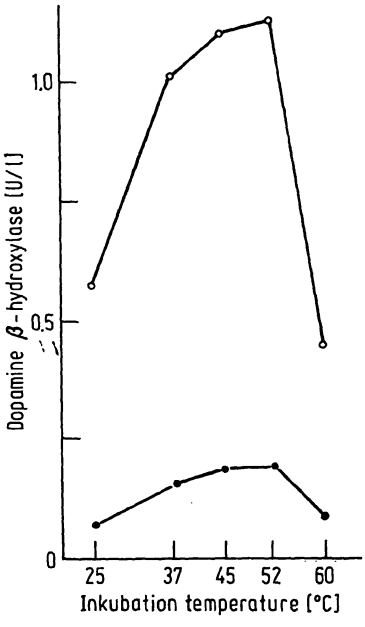

Fig. 5. Variation of serum dopamine β-hydroxylase activity with the incubation temperature (●——● without Cu^{++}; o——o with 25 μmol/l Cu^{++}).

Inhibitory effect of EDTA

EDTA and other chelating agents should inhibit dopamine β-hydroxylase by forming complexes with the enzyme copper. Figure 6 shows this expected effect.

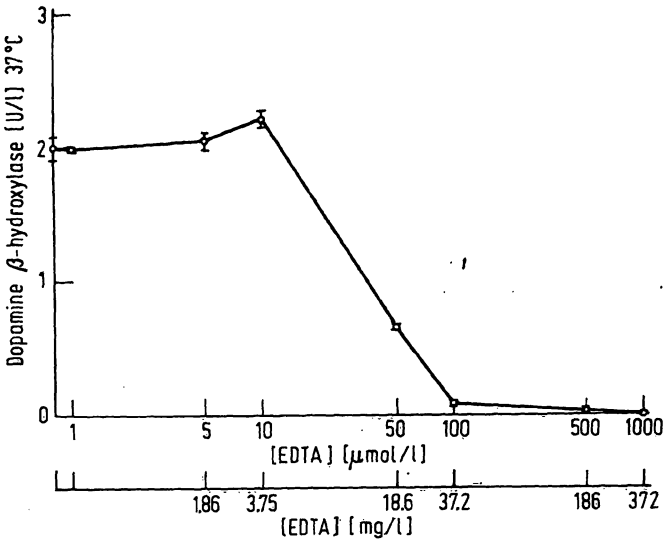

Fig. 6. Inhibition of serum dopamine β-hydroxylase by EDTA.

EDTA concentrations over 10 μmol/l are inhibitory for the enzyme. Thus, blood taken with addition of EDTA, cannot be used for the estimation of the dopamine β-hydroxylase activity.

Discussion

Sub-saturating substrate concentrations are used in this method, because we found it gave greater accuracy. The correlation of the activity measured at 0.05 mmol/l tyramine to that measured at 2.5 mmol/l, tested for 18 sera, gave a linear coefficient r = 0.9694, as determined by linear regression analysis. The dopamine β-hydroxylase activities of these 18 subjects were between 3.58 and 22.87 U/l when 2.5 mmol/l tyramine were used, and between 0.503 and 2.962 units when 50 μmol/l tyramine was present. The ratio of activity at 2.5 mmol/l substrate to activity at 50 μmol/l was 7.38 ± 0.78 (S.D.).

References

1. Kaufman, S. & Friedman, S. (1965), Pharmacol. Rev., *17*, 71–100.
2. Oka, M., Kajikawa, K., Ohuchi, T., Yoshida, H. & Imaizumi, R. (1967), Life Sci., *6*, 461–465.
3. Hörtnagl, H., Hörtnagl, H. & Winkler, H. (1969), J. Physiol. (London), *205*, 103–114.
4. Goldstein, M. & Contrera, J. F. (1962), J. Biol. Chem., *237*, 1898–1902.
5. Goldstein, M., Lauber, E. & McKereghan, M. R. (1965), J. Biol. Chem., *240*, 2066–2072.
6. Friedman, S. & Kaufman, S. (1965), J. Biol. Chem., *240*, 4763–4773.
7. Wallace, E. F., Krantz, M. J. & Lovenberg, W. (1973), Proc. Nat. Acad. Sci. USA, *70*, 2253–2255.
8. Friedman, L. S., Ohuchi, T., Goldstein, M., Axelrod, F., Fish, J. & Dancis, J. (1972), Nature (London), *236*, 310–311.
9. Shopsin, B., Friedman, L. S., Goldstein, M. & Gershon, S. (1972), Psychopharmacologia (Berl.), *27*, 11–16.
10. Rush, R. A. & Geffen, L. B. (1972), Circulation Res. *31*, 444–452.
11. Geffen, L. B., Rush, R. A., Louis, W. J. & Doyle, A. E. (1973), Clinical Science, *44*, 617–620.

Dr. E. S. Markianos
Psychiatrische Klinik der Universität München
Neurochemische Abteilung
D-8000 München 2, Nussbaumstraße 7, Germany